Supporting Information

Materials and Apparatus.

All chemical reagents were obtained from commercial sources and used without further purification unless otherwise noted. Human adenovirus serotype 5 (Ad5) containing luciferase transgene was obtained from Vector Biolabs (Ad5-CMV-Luc) (Philadelphia, PA). Anti-EGFR affibody (ab95116) and Anti-ErbB2 affibody (ab31889) were purchased from Abcam (Cambridge, MA). Succinimidyl-([*N*-maleimidopropionamido]-24ethyleneglycol) ester (NHS-PEG_{24}-Maleimide) was purchased from Thermo Fisher Scientific Inc. (Rockford, IL) and 5-Carboxytetramethylrhodamine, succinimidyl ester from AnaSpec (Fremont, CA). pET15b-Ad12 knob plasmid was kindly donated by Dr. P. Freimuth. Matrix assisted laser desorption-ionization time of flight (MALDI-TOF) mass spectra were obtained on a Bruker Autoflex II mass spectrometer (Berman Germany). HPLC was performed on Shimadzu LG-20AB / SPD-20A system with a C18 column (250 × 4.6 mm, Varian). Size exclusion separation (SEP) columns were purchased from GE Healthcare (NAP-5 column, Sephadex G-25 DNA grade) (Pittsburgh, PA) and SEP spin columns from Princeton Separations (Centri-Sep column CS-400) (Adelphia, NJ). Transmission electron microscope (TEM) image was acquired with a FEI Tecnai BioTwinG^2 and an AMT XR-60 CCD digital camera system. Luciferase activity was measured on a photometer (PerkinElmer 2030 multilabel reader) with luciferase assay kit (Bright-Glo, Promega).

Cell Culture.

Dulbecco's modified Eagle's medium (DMEM) with glutamine, McCoy's 5A medium modified, Penicillin/Streptomycin and 0.5% Trypsin-EDTA were purchased from GIBCO (Grand Island,

NY). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT). A431 (human epithelial carcinoma), PANC-1 (human pancreatic epithelioid carcinoma), MIA PaCa-2 (human pancreatic carcinoma) and RD (human rhabdomyosarcoma) cell lines were cultured in DMEM supplemented with 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin. SK-OV-3 (human ovarian adenocarcinoma) and SK-BR-3 (human breast adenocarcinoma) cell lines were cultured in McCoy's 5A medium containing 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

Metabolic Labeling of Ad5 with *N*-azidoacetylgalactosamine.

Metabolic labeling of Ad5 with *N*-azidoacetylgalactosamine was performed as described previously[1], that is, HEK 293 cells were infected with intact adenovirus particles with a multiplicity of infection (MOI) of 5 PFU/cell. The complete media was supplemented with 50 μM peracetyl-*N*-azidoacetylgalactosamine or 50 μM peracetyl-*N*-azidoacetylglucosamine and the infected cells were incubated at 37 °C. The plates were harvested 42-46 h post infection and virus particles were purified over a gradient of 1.4 g/mL and 1.25 g/mL CsCl centrifuged at 32,000 rpm for 1 h at 15 °C. The virus band at the boundary interface between the two CsCl layers was collected and further purified by an 18 h centrifugation at 35,000 rpm over 1.33 g/mL of CsCl.

Synthesis of maleimide-PEG_{24}-CCH (1).

NHS-PEG_{24}-maleimide (15 mg, 11 μmol) was dissolved in anhydrous acetonitrile and propargylamine (0.6 mg, 11 μmol) was added with 1.5 μL of triethylamine. After 6 h, the reaction mixture was dried *in vacuo* and purified by reverse-phase HPLC with a C18 column and

elution with H_2O/acetonitrile gradient containing TFA (0.1% v/v) to afford **1** (9.2 mg, 63%). MALDI–TOF m/z calcd for $C_{61}H_{111}N_3O_{28}$ [M] 1333.74, obsd 1356.25 $[M + Na]^+$.

Synthesis of Bicyclo[6.1.0]nonyne(BCN)-PEG-maleimide (3).

(1R,8S,9r)-bicyclo[6.1.0]non-4-yn-9-ylmethyl 3,6,9-trioxa-12-azadodecylcarbamate (**2**) was synthesized as described by F. L. van Delft *et al.*[2] **2** (12 mg, 37 μmol) was added to a solution of NHS-PEG_{24}-maleimide (52 mg, 37 μmol) in anhydrous acetonitrile (1 mL) with 1.5 μL of triethylamine. The reaction mixture was stirred for 12 h at room temperature (RT) and concentrated *in vacuo* then purified by HPLC to afford **3** (52 mg, 88%). MALDI–TOF m/z calcd for $C_{75}H_{134}N_4O_{32}$ [M] 1602.90, obsd 1626.28 $[M + Na]^+$.

Conjugation of Affibody with 1.

Affibodies (100 μg, 7.1 nmol) were dissolved in reduction buffer (50 mM sodium phosphate, 150 mM NaCl, 2 mM EDTA, pH 7.5) to obtain a final volume of 0.5 mL. Dithiothreitol (DTT) stock solution (0.5 M) was added to a final concentration of 20 mM DTT and incubated at RT for 2 h. Excess DTT was removed and changed to coupling buffer (PBS, pH 7.2, 5 mM EDTA) by passage through a NAP-5 column, pre-equilibrated with coupling buffer. **1** (1.0 mg, 0.75 μmol) was added immediately after buffer change and the reaction was allowed to proceed for 2 h at RT with gentle mixing. After conjugation, the remaining excess **1** was removed using NAP-5 column with water. MALDI–TOF m/z calcd for anti-EGFR affibody-PEG_{24}-CCH [M] 15185, obsd 15183 $[M + H]^+$; calcd for anti-Her2 affibody-PEG_{24}-CCH [M] 15364, obsd 15367 $[M + H]^+$.

Conjugation of Affibody with 3.

The conjugation of affibody with **3** was performed in the same procedure and condition as with **1**. MALDI–TOF m/z calcd for anti-EGFR affibody-PEG-BCN [M] 15455, obsd 15461 $[M + H]^+$

"Click" Reaction between Azide-labeled Virus (Ad5-GalNAz) and Affibody-PEG_{24}-CCH.

50 μL of azide labeled virus stock (1.0×10^{12} particles/mL) in a 100 mM Tris-HCl buffer pH 8.0 was mixed with bathophenanthroline disulfonic acid disodium salt at a final concentration of 3 mM and affibody-PEG_{24}-CCH at a final concentration of 100 μM. After the mixture was kept in a deoxygenated glove bag for 6 h, copper (I) bromide (CuBr) in dimethyl sulfoxide (DMSO) was added to a final concentration of 1 mM and the reaction was allowed to proceed for 12 h at RT in the deoxygenated glove bag. The reaction was quenched by bathocuproinedisulfonic acid disodium salt (BCS) at a final concentration of 10 mM.

Purification of Modified Viral Particles with Spin Columns.

The viral particles were purified by Centri-Sep columns (CS-400, Princeton Separations, Adelphia, NJ), that is, the columns were gently tapped so that dry gels settle down at the bottom of spin columns and 0.8mL of buffer (PBS + 0.5mM MgCl2 + 0.9mM CaCl2 + 10% glycerol) was added to each column. The column caps were replaced and the gels were hydrated by shaking and inverting or brief vortexing. After 30 min at RT, air bubbles were removed from the top column. The top column caps were removed and excess fluid was drained into wash tube. The columns were spun at 3000 rpm for 2 min. and wash tubes were discarded. 50 μL of modified virus solutions were added and the columns were placed on sample collection tubes. After spinning at 3000 rpm for 2 min., the eluated solutions were collected. The purified "clicked"

viruses were stored in PBS buffer with 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ containing 10% glycerol and kept in 4 ˚C for infection test. The purified viruses can be stored in 4 ˚C up to 2 weeks.

Quantitation of Viral Particles by PicoGreen Assay.

The purified viruses were quantitated using PicoGreen reagent (Quant-iT, invitrogen, Carlsbad, CA) as described by M. T. McCaman *et al.*[3] Briefly, 0.5 μL of 0.5 % SDS TE buffer was added to 4.5 μL of virus solution and incubated at 37 ˚C for 30 min. This virus lysis solution was diluted with 45 μL of TE. Working solution was prepared by adding 25 μL of Quant-iT PicoGreen in 4.975 mL of TE. 20 μL of diluted lysis solution and 180 μL of working solution was mixed and analyzed by fluorescence photometer. Using standard double stranded DNA, the fluorescence (excitation: 485 nm, emission: 538 nm) standard curve (y = ax + b; x = DNA ng, y = fluorescence intensity) was obtained. The number of viral particle was calculated using the standard curve and a conversion factor (1 ng DNA = 2.6×10^7 Ad genome, i.e., Ad particles).

Synthesis of Tetramethylrhodamine-BCN (4) (TAMRA-BCN).

5-Carboxytetramethylrhodamine, succinimidyl ester (2.5 mg, 4.7 μmol) was dissolved in anhydrous DMF and **2** (1.5 mg, 4.7 μmol) was added with 1.5 μL of triethylamine at RT. After 6 h, the reaction mixture was dried *in vacuo* and purified by reverse-phase HPLC with a C18 column and elution with H_2O/acetonitrile gradient containing TFA (0.1% v/v) to afford **4** (2.8 mg, 80%). MALDI–TOF m/z calcd for $C_{42}H_{48}N_4O_8$ [M] 736.35, obsd 737.60 $[M + H]^+$.

Fluorescent Gel Scanning Assay with TAMRA-BCN.

Azide enabled viral particles (Ad5-GalNAz) were labeled with an TAMRA-BCN dye using strain promoted "Click" chemistry, that is, TAMRA-BCN (20 mM) was added to GalNAz labeled Ad stock (1.0×10^{12} particles/mL) to obtain final concentration of 100 μM TAMRA-BCN and the reaction was allowed to proceed for 2 h at RT. After "Click" reaction viral particles were purified using Centri-Sep spin columns and quantitated with Quant-iT PicoGreen as described above. The purified viral stock (1×10^{12} viral particles/mL) was run on SDS-PAGE using the TAMRA-BCN dye as standard. Standard dye was loaded 15 min before the end of the run. Gels were scanned using a typhoon gel scanner in fluorescence mode with excitation filter at 532 nm and emission filter at 580 ± 15 nm. The fluorescence intensities of scans were subsequently analyzed with Image Quant TL 1D gel analyzer software. All gels were run at 4 °C for 60 min and scanned within 10 min after the end of run.

"Click" Reaction between Azide-labeled Virus (Ad5-GalNAz) and Affibody-PEG-BCN.

100 μL of azide labeled virus stock (1.0×10^{12} particles/mL) in a PBS buffer pH 7.4 was gently mixed with affibody-PEG-BCN (100 μM) for 16 h at RT using LabQuake tube shaker. The viral particles were purified, quantitated and stored with the same method as described above ("Click" reaction between Ad5-GalNAz and Affibody-PEG_{24}-CCH).

Western Blotting for Viral Fiber.

To all samples (10^9 viral particles of unmodified Ad, Affibody (anti-EGFR) conjugated Ads through Cu (I) promoted and strain promoted chemistry, respectively), loading dye was added and boiled at 95 °C for 10 min. The samples were run on a 10% polyacrylamide electrophoresis gel at 100 V for 3 h and transferred onto nitrocellulose membrane at 40 V over 2 h in a western

transfer buffer (25 mM tris, 192 mM glycine, 0.5% SDS and 10% methanol). Blots were blocked by 5% skim milk in PBST for 1 h at RT on rocker and washed with PBST twice, then treated with primary α-Fiber (Ad5) rabbit antibody (kindly donated by Dr. Patrick Hearing, Stony Brook university) at a ratio of 1:1000 in 3% BSA for 1 h. Blots were washed 3 times with 5% skim milk and treated with secondary α-rabbit HPR conjugate antibody (GE Healthcare, Cat: NA934) at a ratio of 1:5000 with 5% skim milk in PBST for 1 h. The membrane was washed 3 times with 5% skim milk in PBST and twice with PBST. The chemiluminescent HPR substrate (Millipore Immobilon Western, Bilerica, MA) were introduced to the membranes for 2 min and the membranes were exposed to film in dark room for 20 sec.

TEM Image of Virus. Approximately 15μL of purified virus stock was placed on 400 mesh formvar coated copper grids for 1 min, fixed with 2% EM grade glutaraldehyde for 1 min and then counter stained with 2% phosphotungstic acid for 30 sec. Samples were then viewed with a FEI Tecnai BioTwinG^2 transmission electron microscope. Digital images were acquired with an AMT XR-60 CCD digital camera system and compiled using Adobe Photoshop.

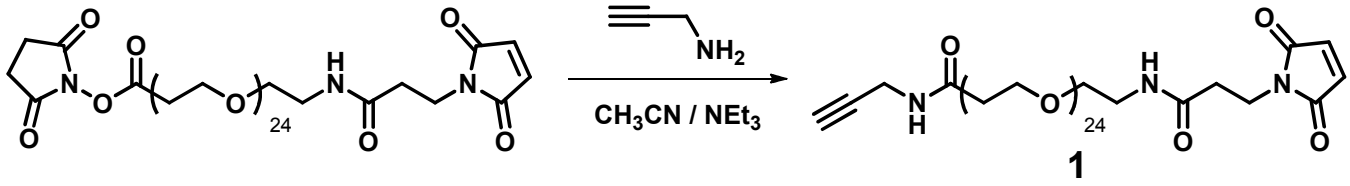
Thermal Stability Assay of Virus. Thermal stability of virus was assayed as described by C. R. Middaugh[4] using propidium iodide (PI). Briefly, the fluorescence spectra (excitation at 535 nm, emission scan from 550 to 650 nm, λ_{max} = 613, slit widths of 6 nm) of PI (7.5 μM) were collected at the viral concentration of 6.5×10^{10} particles /mL in 5 ˚C increments from 20 to 90 ˚C employing a QuantaMaster spectrofluorometer (Photon Technology International, Inc.) with a 5 min temperature equilibration time before data acquisition. The T_m was determined by

calculating the point where the second derivatives of the λ_{max} versus temperature plots crossed the x-axis using Microcal Origin.

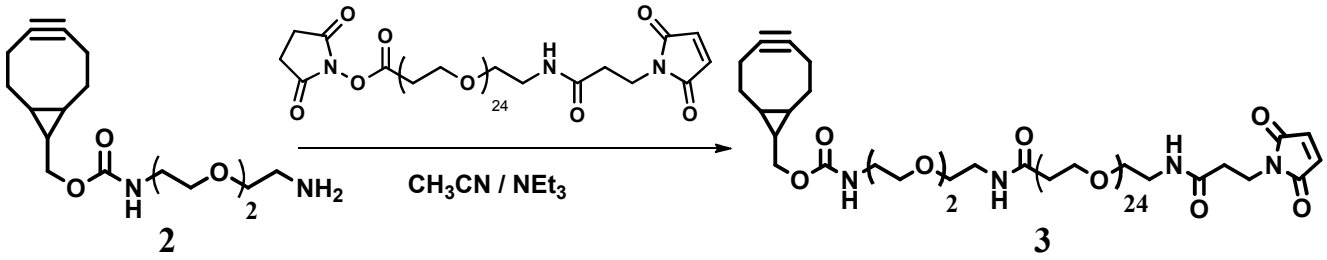
Expression and Purification of Ad12 Knob. Ad12 knob was expressed and purified as described by P. Freimuth *et al.*[5].

Infection Assay. Cells (A431, PANC-1, MIA PaCa-2, RD, SK-OV-3 and SK-BR-3) were seeded in 96-well black and clear bottom plates at a density of 1×10^4 cells/well, respectively, 1 day before infection. Monolayer cells were washed with PBS and incubated in inhibition buffer ([Ad12 knob] ≈ 20 μM in PBS with 2% serum) for 1 h at 37 °C. Virus stock was added to the inhibition buffer at a multiplicity of infection (MOI) of 1000 and incubated at 37 °C. After 1 h, cell growth media were added and cells were incubated at 37 °C. 24 h post infection, the expression levels of luciferase transgene were determined by a photometer with the luciferase assay kit (Bright-Glo, Promega).

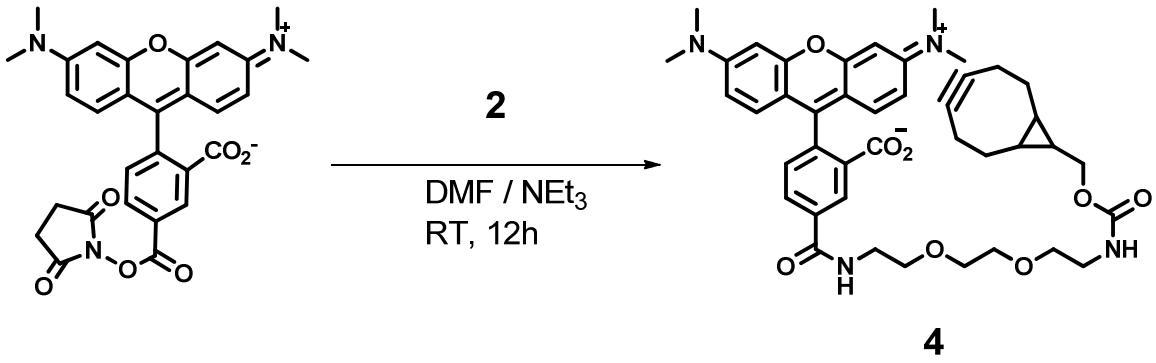
Statistics. Experiments were performed in triplicate and presented as mean ± standard deviation. *P*-values were calculated by a two-tailed unpaired Student's t-test. Data were considered to be significantly different when $P < 0.05$.



Scheme S1. Synthesis of maleimide -PEG_{24}-CCH (**1**)



Scheme S2. Synthesis of maleimide -PEG-BCN (**3**)



Scheme S3. Synthesis of TAMRA-BCN (**4**)

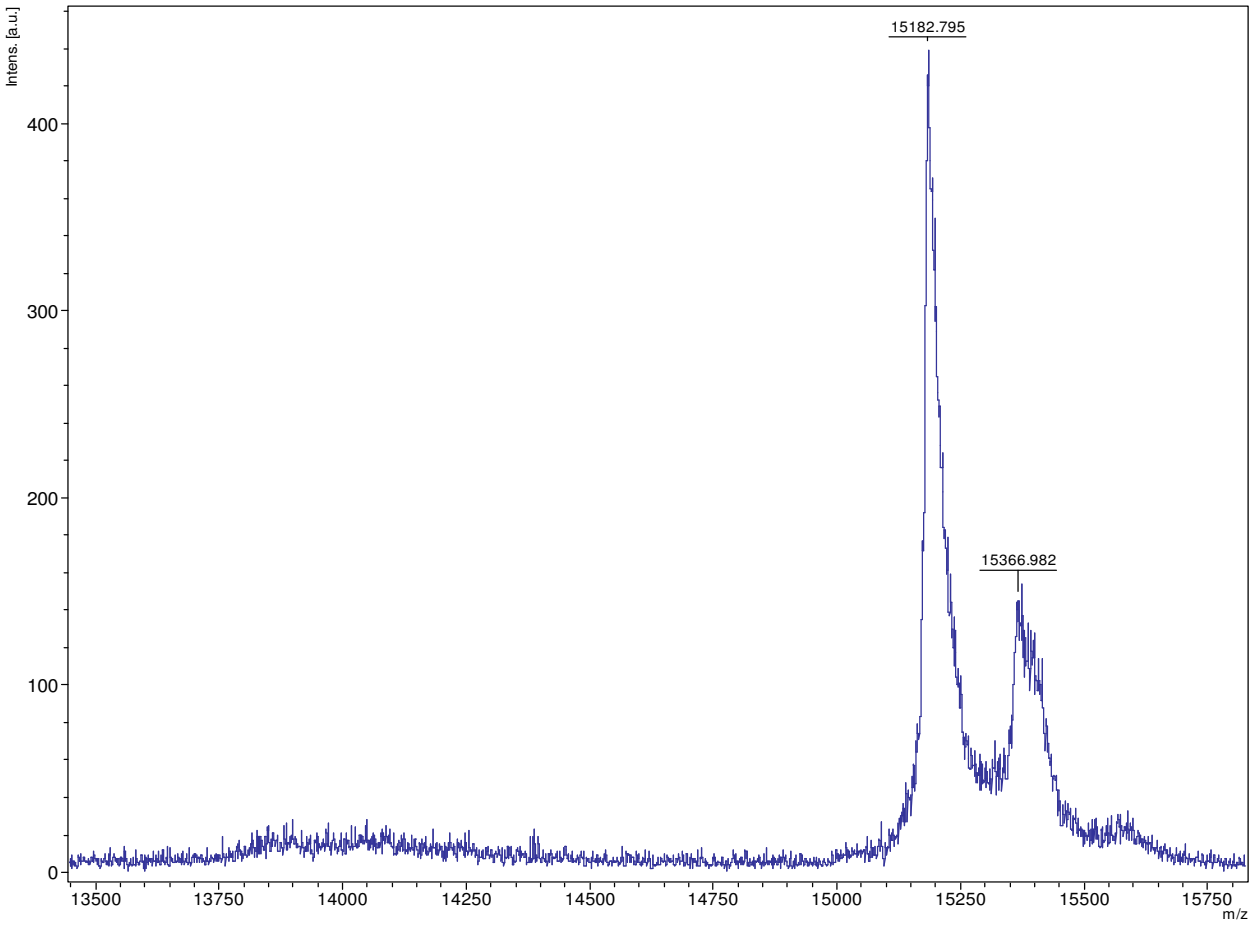


Figure S1. MALDI-TOF spectrum of affiEGF-PEG_{24}-CCH (Matrix: CHCA) m/z calcd [M] 15185, obsd 15183 $[M + H]^+$,

the m/z calcd is monoisotopic mass based on the amino acid sequence of anti-EGFR affibody (GSSLQVDNKFNKEMWAAWEEIRNLPNLNGWQMTAFIASLVDDPSQSANLLAEAKKLN DAQAPKVDNKFNKEMWAAWEEIRNLPNLNGWQMTAFIASLVDDPSQSANLLAEAKKL NDAQAPKVDC).

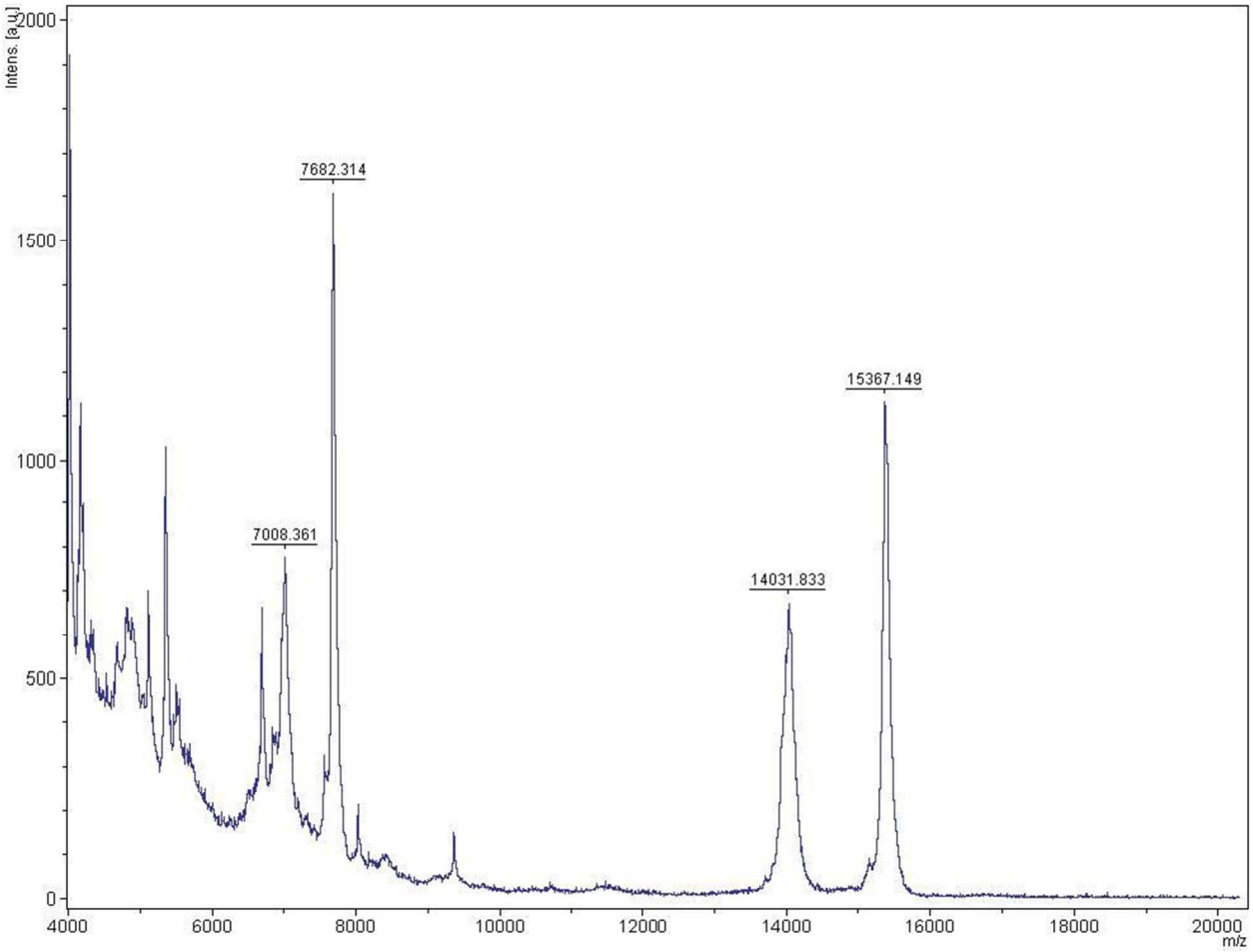


Figure S2. MALDI-TOF spectrum of affiHer2-PEG_{24}-CCH (Matrix: CHCA), m/z calcd [M] 15364, obsd 15367 $[M + H]^+$, the m/z cald is monoisotopic mass based on the amino acid sequence of anti-Her2 affibody (GSSLQVDNKFNKEMRNAYWEIALLPNLNVAQKRAFIRSLYDDPSQSANLLAEAKKLND AQAPKVDNKFNKEMRNAYWEIALLPNLNVAQKRAFIRSLYDDPSQSANLLAEAKKLND AQAPKVDC). Unconjugated affibody (m/z 14032) was removed by a size exclusion column (Mw cut-off: 100 KDa) after "click reaction" with virus.

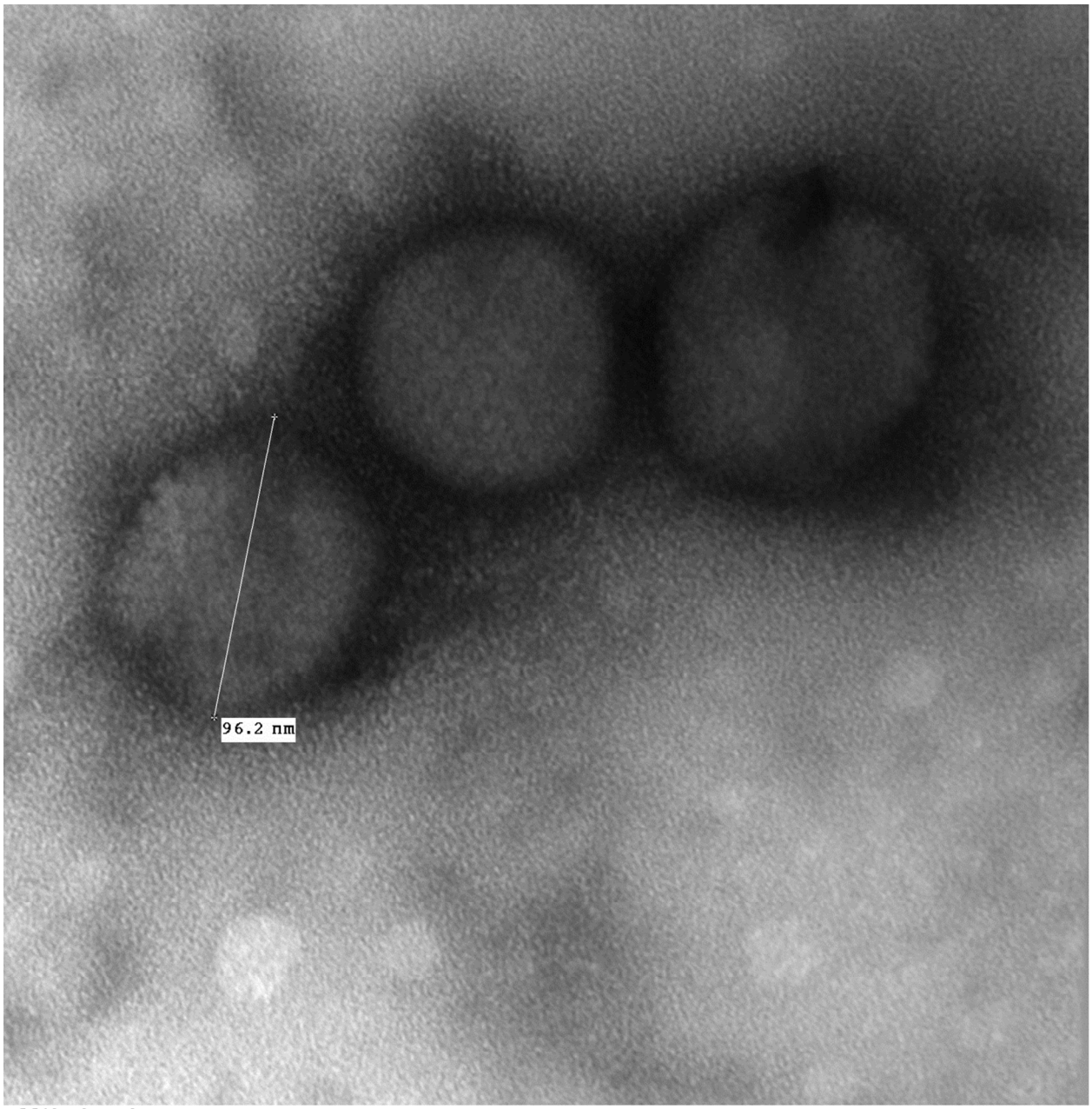


Figure S3. TEM image of affiEGF-PEG_{24}-Ad, fixed with 2% EM grade glutaraldehyde for 1 min and then counter stained with 2% phosphotungstic acid for 30 sec.

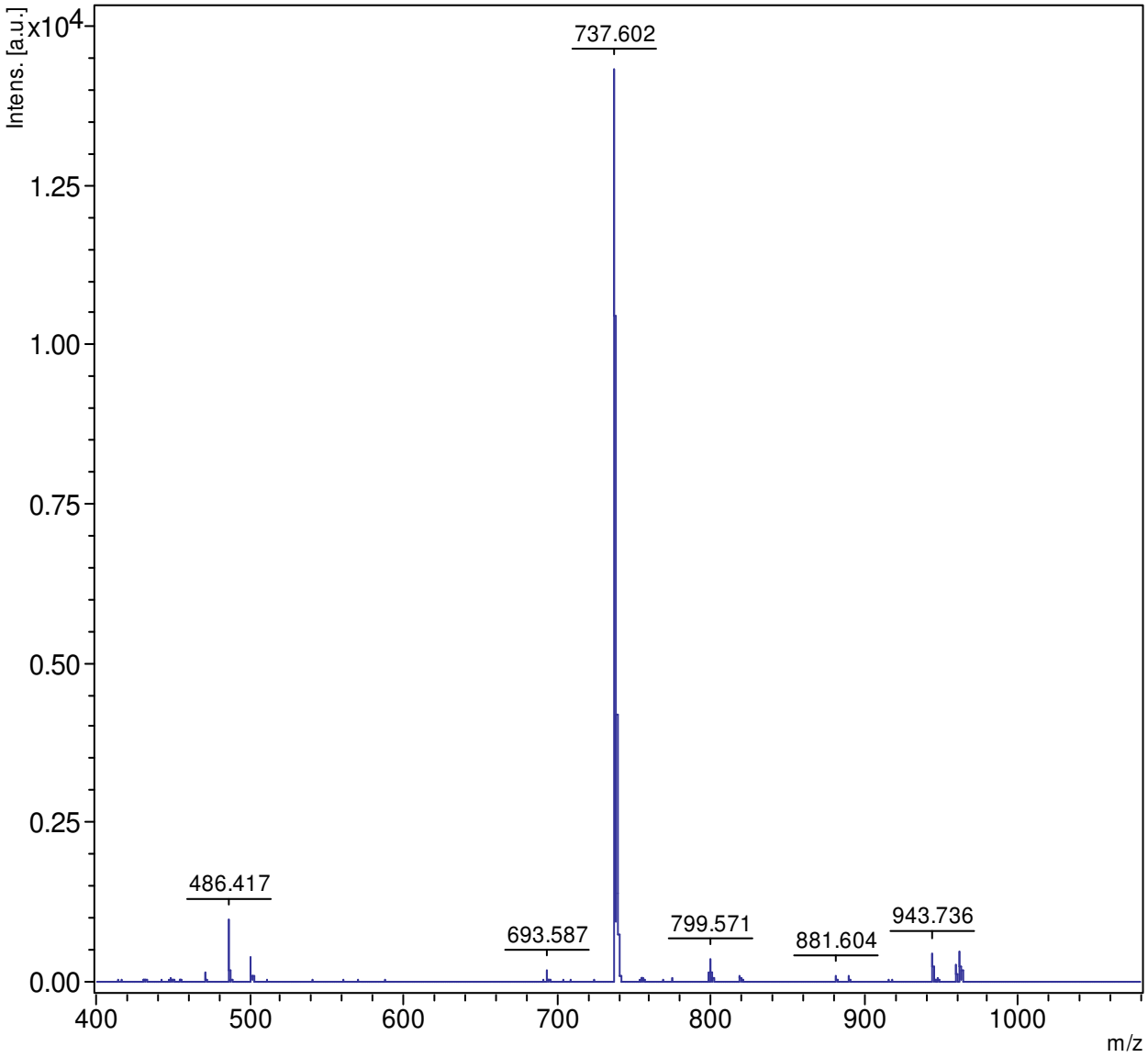
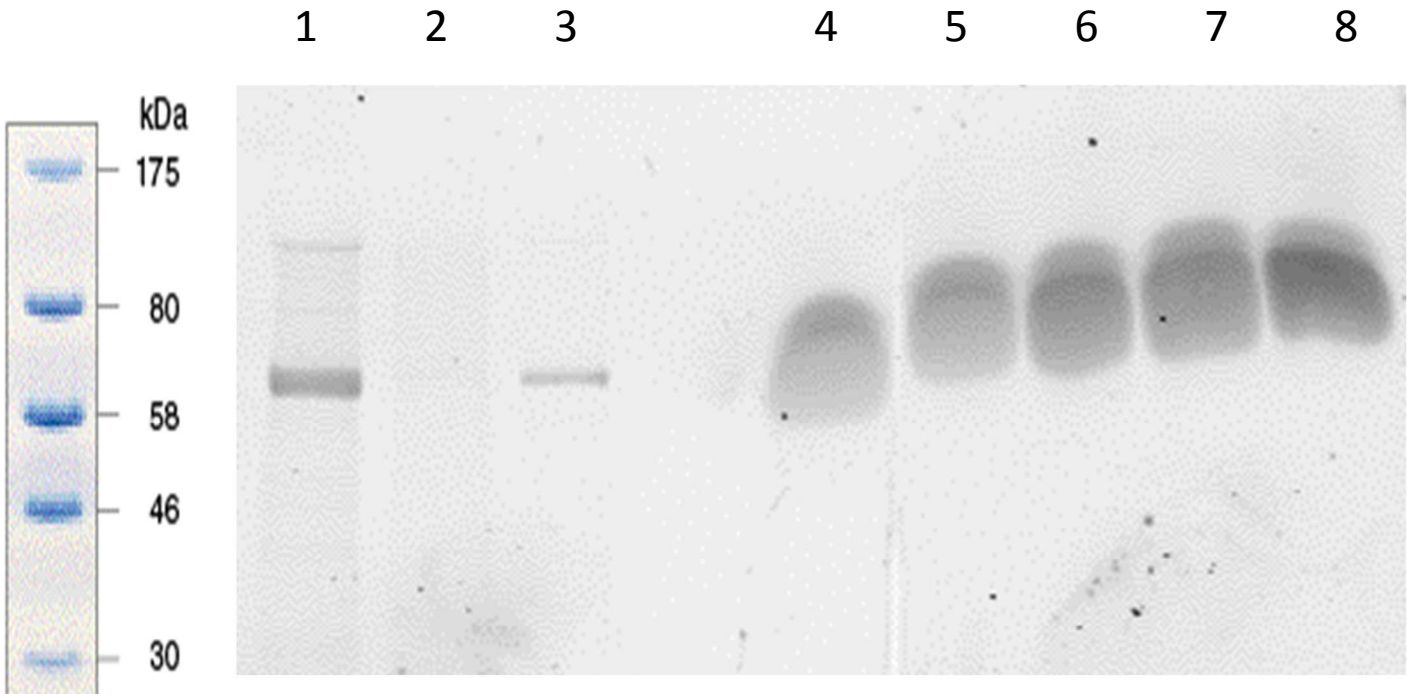


Figure S4. MALDI-TOF spectrum of TAMRA-BCN (**4**), m/z calcd for $C_{42}H_{48}N_4O_8$ [M] 736.35, obsd 737.60 $[M + H]^+$.



[TAMRA-BCN]	100 µM	100 µM	100 µM	0.4 nM	0.5 nM	1.0 nM	1.5 nM	2.0 nM
Virus	GalNAz-Ad	Intact Ad	GalNAz-Ad	-	-	-	-	-
Dialysis	-	-	+	-	-	-	-	-

Figure S5. SDS-PAGE fluorescent assay for quantitation of stain promoted modification of GlcNAz labeled adenovirus and TAMRA-BCN. lane1: TAMRA-BCN (20 mM) was added to GalNAz labeled Ad stock (1.0×10^{12} particles/mL) to obtain final concentration 100 µM of TAMRA-BCN and the reaction was allowed to proceed for 2 h at RT; lane2: reaction was performed at the same conditions as lane 1 except using intact Ad (negative control); lane3: to support covalent conjugation between TAMRA-BCN and viral fiber of GalNAz-Ad, adenoviral

fiber was partially purified from lane 1 reaction mixture, i.e., TAMRA-BCN-GlcNAz labeled adenoviral particles were dialyzed overnight in a Tris-maleate (5 mM Tris, 5 mM maleic acid, 1 mM EDTA) buffer at pH 6.5. The dialyzed solution was centrifuged at 14,000 rpm for 1 h at 4 ˚C, after which the supernatant containing the labeled fiber proteins was separated from the precipitated viral capsids and run on the gel; lane 4~8: TAMRA-BCN standard solutions with different concentrations.

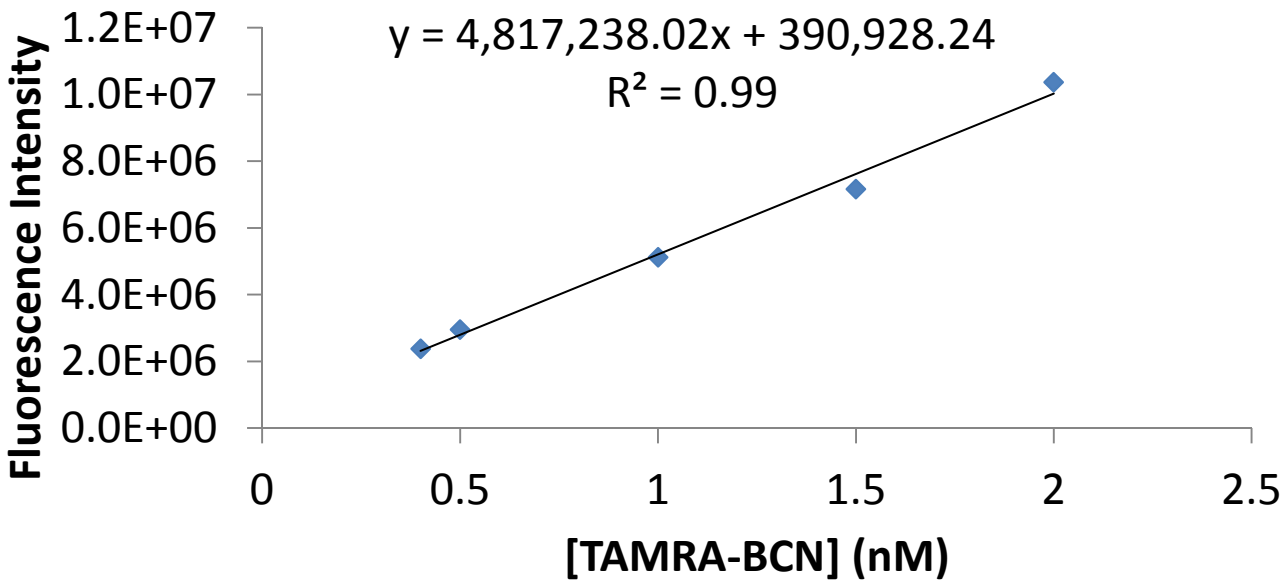


Figure S6. Standard calibration curve for determination of [TAMRA-BCN] conjugated on viral particles.

Reaction time (h)	Concentration of dye on 10^{12} viral Fiber (nM)	Dye/ viral particle
2	1.69	1.01
16	14.7	8.84

Table S1. Column 1 shows reaction (strain-promoted alkyne-azide cycloaddition) time at RT; Column 2, the concentration of dye on viral fiber calculated from fluorescence gel scans and the standard calibration curve; column 3 shows the corresponding number of dye molecules per viral particle deduced from column 2 and the molarity of viral particles (1.00×10^{12} particles/ mL $\approx$ 1.66 nM)

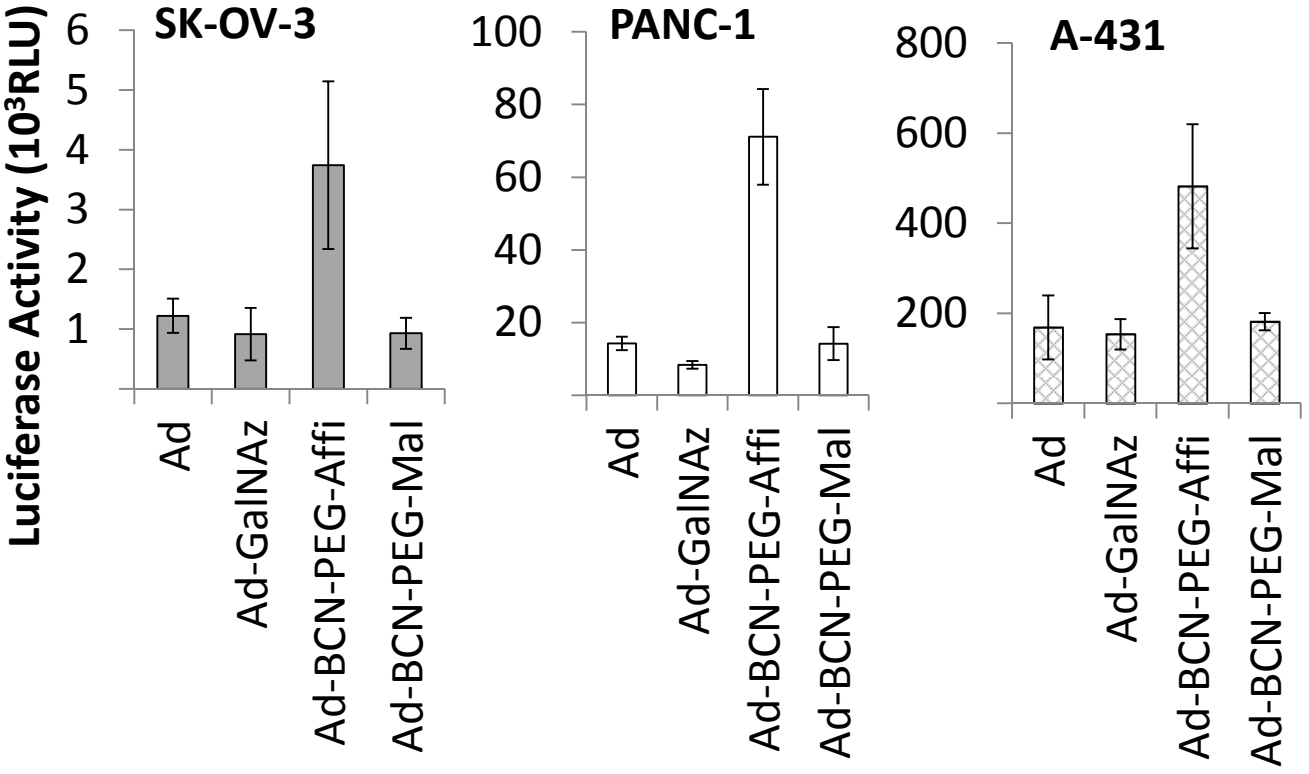


Figure S7. Transduction assay with affiEGF-PEG-BCN-AdLUC, 2×10^4 cells/well, CAR inhibition with [Ad12 knob] ≈ 20 μM for 1 h at 37 °C, 1000 MOI, 24 h, Ad: intact AdLUC, Ad-GalNAz: *O*-GlcNAz enabled AdLUC, Ad-BCN-PEG-Affi: anti-EGFR affibody-PEG-BCN-AdLUC, Ad-BCN-PEG-Mal: maleimide-PEG-BCN-AdLUC (Ad w/o targeting group as a negative control) ($P < 0.05$).

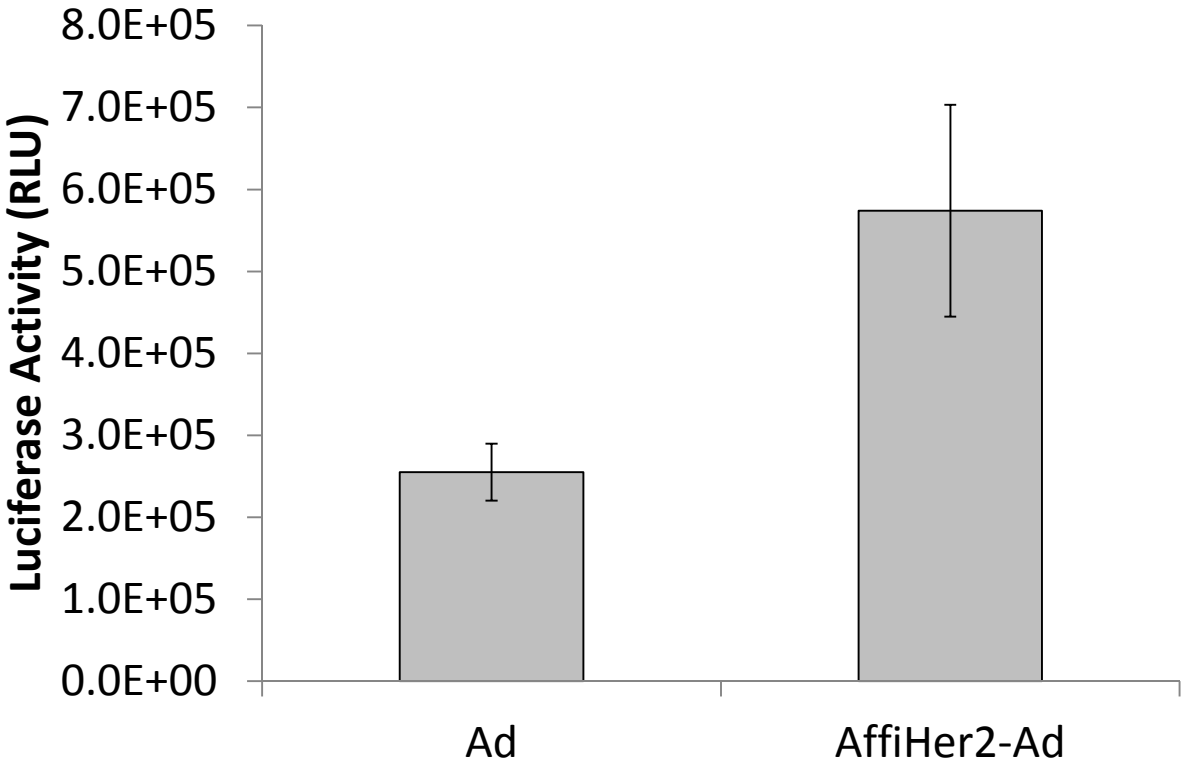


Figure S8. Transduction assay with affiHer2-PEG-AdLUC without CAR inhibition, SK-BR-3 human breast cancer, 2×10^4 cells/well, 1000 MOI, 24 h infection, Ad: intact AdLUC without Ad12 knob, AffiHer2-Ad: affiHER2-PEG-CCH conjugated Ad without Ad12 knob

References

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